

Genomic fingerprinting of penicillinase-producing strains of *Neisseria gonorrhoeae* in Valencia, Spain

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Abstract

Objective—To compare the value of different markers and their combinations with the restriction enzyme technique in the differentiations of penicillinase-producing *N. gonorrhoeae* (PPNG) strains.

Materials and methods—17 PPNG strains isolated from symptomatic, untreated male patients with urethritis were characterised by antibiotic sensitivity testing, auxotyping, serotyping, plasmid profile, and restriction endonuclease fingerprinting (Hind III digestion). Cluster analysis with the method of unweighted pair-group average (UPGMA) linkage was used to calculate similarity or dissimilarity for PPNG strains.

Main Results—Either auxotyping or plasmid profile alone differentiated three groups of PPNG strains, whereas the combination auxotyping/serotyping identified 10. Although the combination auxotyping/serotyping/plasmid profile and the restriction enzyme technique showed a similar discrimination ability (differentiation of 11 PPNG strains), genomic fingerprinting gave highly specific restriction patterns on individual gonococcal isolates.

Conclusions—The combination of different markers gave more epidemiological information than the use of only one. The sequence of discriminating ability for PPNG strains was: auxotyping/serotyping < auxotyping/serotyping/plasmid profile < restriction patterns of genomic DNA.

Introduction

The incidence of penicillinase (beta-lactamase)-producing strains of *Neisseria gonorrhoeae* has been steadily increasing in most European countries, including Spain.¹ Phenotypic and genotypic characterisation of these strains has proved valuable for epidemiological tracing in gonococcal disease. Auxotyping,² antibiotic susceptibility testing,³ serogrouping,⁴⁻⁹ plasmid analysis, and the so-called genomic fingerprinting based on restriction endonuclease digestion of the gonococcal DNA,¹⁰⁻¹¹ have been used by different research groups as epidemiological tools for differentiating between penicillinase-producing *N. gonorrhoeae* strains. Genomic fingerprinting gives highly reproducible, stable, and specific restriction patterns on individual gonococcal isolates¹²⁻¹⁶ and can serve as a useful adjunct to serological classification.

We compared the value of different markers and their combinations with the restriction enzyme technique in the differentiation of penicillinase-producing strains of *N. gonorrhoeae*.

Materials and methods

Gonococcal strains

A total of 17 penicillinase-producing *N. gonorrhoeae* strains isolated from symptomatic, untreated male patients with urethritis in Valencia, Spain, between 1985 and 1988 were included in the study. They accounted for 9.9% of all isolates of *N. gonorrhoeae* during this period.

All isolates were confirmed as *N. gonorrhoeae* by colony morphology, Gram stain, oxidase reaction, and acid reproduction from glucose but not maltose, sucrose, lactose or fructose. Purified stock cultures were maintained in trypticase-soy broth (BBL Microbiology Systems, Cockeysville, MD, USA) containing 20% glycerol and stored at -70°C . Isolates were subcultured on GC agar medium base (Oxoid, UK) with 1% (v/v) haemoglobin (BBL), 1% (v/v) IsoVitallex (BBL) and without antibiotics, and incubated at 37°C with 5% CO_2 atmosphere in a humidified incubator for 20 h. The strain number was that assigned in our laboratory.

The isolates were tested for β -lactamase production by means of the chromogenic cephalosporin test.¹⁷

Antibiotic sensitivity testing

We tested sensitivity to antibiotic by an agar dilution method³ using GC agar base enriched with 1% (v/v) IsoVitallex containing various concentrations (0.125 to 128 mg/l) of penicillin G; 10^4 colony forming units (cfu) were transferred to the agar surface. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that completely suppressed bacterial growth after 18–24 h incubation.

Auxotyping

Auxotyping was performed by growing *N. gonorrhoeae* on chemically defined medium as described previously.² Strains were tested for their requirements for proline (Pro^-), arginine (Arg^-), methionine (Met^-), hypoxanthine (Hyx^-), and uracil (Ura^-) or combinations of these requirements. Strains with no special requirements regarding these substances were called prototrophic (Proto).

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Table Distribution of 17 penicillinase-producing *Neisseria gonorrhoeae* strains regarding minimum inhibitory concentrations (MIC), plasmid size, auxotype, and serogroup and serovar patterns.

Strain designation	MIC (mg/l)	Plasmid size (megadaltons)	Auxotype	Serogroup/serovar
20	32	3.2	Arg	IA/rst
44	64	4.5	Proto	IB/rop
52	>128	4.5	Proto	IB/rop
64	64	3.2	Proto	IB/x
76	16	3.2	Proto	IB/o
93	64	4.5 and 24.5	Pro	IB/rop
96	>128	3.2	Pro	IB/pyvut
99	16	3.2	Proto	IB/x
102	>128	3.2	Pro	IB/opt
103	128	3.2	Pro	IB/pu
115	>128	3.2	Pro	IB/pyvut
136	64	4.5	Proto	IB/rop
167	128	3.2	Pro	IB/pyvut
171	8	3.2	Proto	IB/rop
180	128	4.5	Proto	IB/rop
185	128	3.2	Pro	IB/pyvut
186	>128	3.2	Proto	IA/v

Plasmid analysis

Plasmids were isolated by a rapid alkaline extraction procedure as described by Birnboim and Doly,¹⁸ with some modifications.¹⁹ Plasmids were analysed in 0.8% agarose gels. The *Escherichia coli* V517 strain²⁰ (size estimates of plasmids 1.8, 2.0, 2.6, 3.4, 3.7, 4.8 and 35.8 10⁶ daltons) and *N. gonorrhoeae* strains CCUG 5449 (size estimates of plasmids 3.2 and 2.6 10⁶ daltons) and CCUG 6016 (size estimates of plasmids 24.5, 4.5 and 2.6 10⁶ daltons) (Culture collection, University of Göteborg, Sweden) with plasmids of known molecular weight were included as fragment size markers.

Serological classification

Serological classification into serogroups (WI, WII, and WIII) and serovars was performed by co-agglutination as described previously^{4, 21} using a set of monoclonal antibodies bound to staphylococci containing protein A (provided by S Bygdeman, Huddings University

Hospital, Stockholm, Sweden). Each strain was tested against a set of five antibodies specific to protein AI (designated Ar, Ao, As, At, and Av) and nine antibodies specific to protein IB (Br, Bo, Bp, By, Bv, Bu, Bs, Bt, and Bx).

Penicillinase-producing strains of *N. gonorrhoeae* were distributed into serovars according to their reactions with the different monoclonal antibody reagents. The capital letters A (for WI strains) and B (for WII/WIII) were followed by lower case letters representing positive reactions with the corresponding coagglutination reagents.

Restriction endonuclease fingerprinting

The restriction enzyme technique was performed as described by Falk *et al.*¹⁰ with some modifications. The restriction endonuclease used was Hind III (Boehringer GmbH, Mannheim, Germany). After electrophoresis, gels were stained with silver (Bio-Rad Lab., Richmond, CA, USA) or alternatively with ethidium bromide. Electrophoretograms were scanned with an LKB2202 UltraScan laser densitometer (Pharmacia LKB, Uppsala, Sweden) provided with a Hewlett-Packard 3390 integrator. The molecular weight of each digested DNA fragment was calculated by a computer program of robust estimation described by Plikaitis *et al.*²²

Statistical methods

Statistical analyses of data for grouping different isolates were carried out by assigning to each electrophoretogram a dimensional vector "n" of presence/absence (presence = 1, absence = 0), where "n" was the total number of resulting DNA fragments. These vectors were introduced into a program of cluster analysis using the "proximities" procedure²³ for estimating the distance matrix (SM) according to the following equation: SM (x, y) = a + d/a + b + c + d, where SM (x, y) was the similarity coefficient between two strains; "a" the number of DNA fragments which were present in both strains; "b" the number of DNA fragments which were present in the "x" strain and absent in the "y" strain; "c" the number of DNA fragments which were present in the "y" strain and absent in the "x" strain; and "d" the number of DNA fragments which were absent in both strains. Once this matrix has been calculated, the cluster analysis with the method of unweighted pair-group average (UPGMA) linkage was used. In order to obtain a graphic representation (dendrogram), the program used converted the similarity coefficients [SM (x, y)] into RD (x, y) (rescaled distance cluster combine) according to the following formula:

$$RD(x, y) = \frac{1 - SM(x, y)}{SM_{max} - SM_{min}} \times N$$

where, "Smax" and "Smin" were the values of the maximum and minimum similarity coefficients and "N" the index of the scale. Analyses were carried out with SPSS²⁴ running on a Honeywell Bull computer.

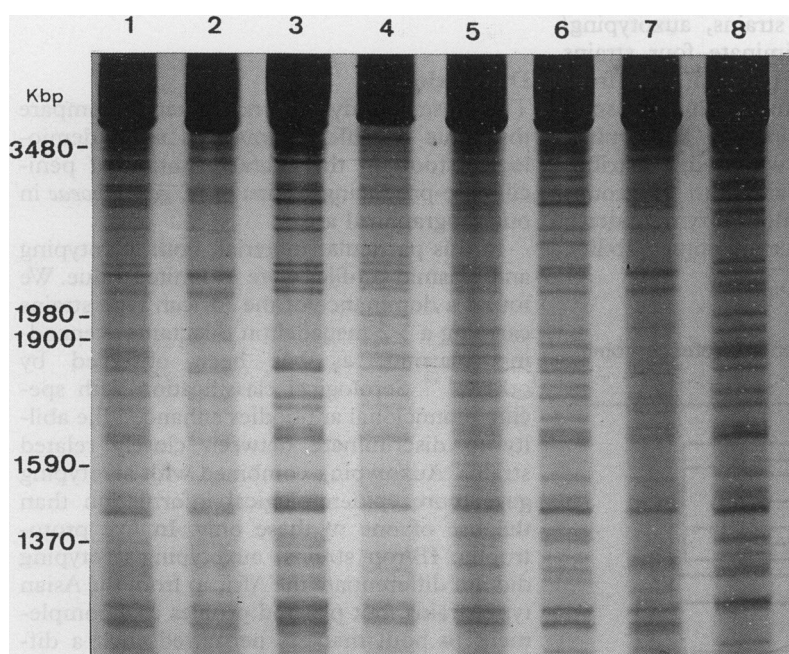


Fig 1 Genetic heterogeneity of *N. gonorrhoeae* strains after DNA digestion with Hind III (lane numbers 1-8 corresponding to strains 52, 171, 102, 93, 185, 20, 186, and 103). bp, base pairs.

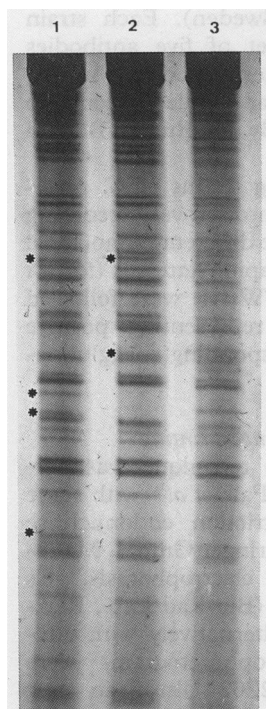


Fig 2 The restriction enzyme patterns of strains 99 and 64 (lanes 1 and 2, respectively) with identical plasmid size, auxotype and serovar patterns differed only with regard to 2 or 4 bands; *presence/absence of bands (silver stain).

Results

All isolates were β -lactamase producers with MIC values of penicillin ranging between 8 and >128 mg/l (table). Three different auxotypes were identified. Nine of the 17 strains were Proto, seven were Pro⁻, and only one was Arg⁻ (table).

The 3.2 megadalton or Africa (Af⁻) plasmid was found in 12 (70.6%) strains. Four strains contained the 4.5 megadalton or Asia (As⁻) plasmid. In one strain the conjugative 24.5 megadalton plasmid coexisted with the 4.5 megadalton plasmid (As⁺). The cryptic 2.6 megadalton plasmid was present in all 17 strains (table).

Serogroup WI was represented by two isolates which belonged to the serovars Arst and Av. Serogroup VII/WIII was represented by 15 strains belonging to six different serovars (table). The serovar pattern Brop was identified in 35% of the isolates.

Restriction patterns obtained with Hind III digestion of genomic DNA of different isolates consisted of a mean of 50 bands per pattern of which size of fragments between 2.4 and 1.3 kilobase pairs (containing a median of 25 to 30 bands) were studied (fig 1). Three common bands with fragment sizes of 2.1, 1.5, and 1.3 kilobase pairs, respectively, were found in all lanes. Highly resolved bands standing out minimal differences between two isolates were obtained with silver stain (fig 2).

Statistical grouping of isolates showed similarity coefficients between 1.0 and 0.7456 [RD (x, y) 0 and 17, respectively]. With a cutoff level at rescaled distance cluster [RD (x, y)] of 8, four clearly defined clusters of *N. gonorrhoeae* strains were identified. Figure 3 shows the grouping dendrogram.

Discrimination by different markers and their combinations is shown in fig 4. Auxotyping differentiated three groups of strains of *N. gonorrhoeae*, whereas the combination auxotyping/serotyping differentiated 10. Among the five prototrophic IB/rop strains, auxotyping/serotyping did not discriminate four strains with the 4.5 megadalton plasmid (As⁻) from one strain with the 3.2 megadalton plasmid (Af⁻). When the combination auxotyping/serotyping/plasmid profile was used, a distribution of *N. gonorrhoeae* strains into 11 groups was obtained. The restriction enzyme pattern showed a similar discrimination ability,

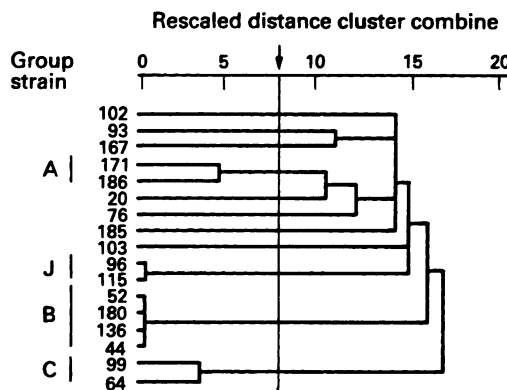


Fig 3 Dendrogram of penicillinase-producing *N. gonorrhoeae* strains. Clusters of strains (a, b, c, j) at the 8 cutoff level.

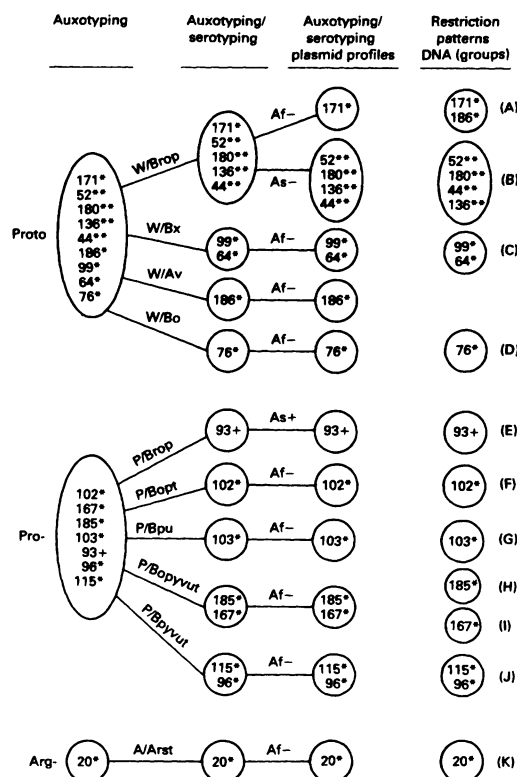


Fig 4 Phenotypic and genotypic discrimination of 17 penicillinase-producing *N. gonorrhoeae* strains. Auxotypes: Proto (prototrophic), Pro (proline), Arg (arginine). Plasmid profile and groups obtained with DNA restriction patterns, *3.2 megadalton plasmid (Af⁻), **4.5 megadalton plasmid (As⁻), †24.5 and 4.5 megadalton plasmid (As⁺).

although it detected differences between two Af⁻ strains (isolates 185 and 167) which were completely identical by combining the other markers. However, the restriction enzyme pattern was unable to differentiate isolates 171 and 186 (fig 4).

Discussion

The present study was undertaken to compare the value of different markers as epidemiological tools in the characterisation of penicillinase-producing strains of *N. gonorrhoeae* in our geographical area.

In this particular material, both auxotyping and plasmid profiles were of limited value. We found a dominance of the African type strains carrying a 3.2 megadalton β -lactamase encoding plasmid, as has been observed by others.^{1,14} Serological classification with specific monoclonal antibodies enhanced the ability to discriminate between closely related strains. Auxotyping combined with serotyping gave more epidemiological information than the use of one of these only. In five prototrophic IB/rop strains, auxotyping/serotyping did not differentiate the African from the Asian type strains, but plasmid profiles as a complement to both markers permitted such a differentiation.

The distribution of gonococcal strains into 11 groups obtained with the combination of

three markers (auxotyping/serotyping/plasmid profiles) was in agreement with the genomic fingerprinting. The restriction enzyme technique may differentiate between strains that by phenotypic classification appear to be identical. The reproducibility and reliability of the method has been demonstrated by other investigators.^{13-16 25} Moreover, the use of silver stain has been proved a valuable alternative to ethidium bromide.

The characterisation of a clone as As⁺ IB/rop strains may have been linked to an outbreak that occurred in our urban area at the end of 1987. The two Af⁻ IB/x strains in which all markers and their combinations coincided, were similar to those described in an outbreak of penicillinase-producing strains of *N. gonorrhoeae* in the city of Barcelona (close to Valencia) in 1987.²⁶

The combination of different markers has been shown to give more epidemiological information than the use of only one. Although the restriction enzyme technique appears to be highly specific, it is both laborious and expensive and usually limited to special laboratories. We conclude that the combination of serological classification with specific monoclonal antibodies with auxotyping and/or plasmid profiles is of great potential use for epidemiological and clinical studies. Genotypic fingerprinting could be of practical importance for detecting subtle genetic differences of phenotypically similar gonococcal clones.

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